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**ANTONELLA FUSILLO**

(Name of person mailing paper or fee)

(Signature)

**TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371**

Attorney's Docket No:  
**SEIFARTH**

INTERNATIONAL APPLICATION NO.

**PCT/DE00/01071**

INTERNATIONAL FILING DATE

**4 April 2000**

PRIORITY DATE CLAIMED

**8 May 1999**

TITLE OF INVENTION

**METHOD FOR THE SPECIFIC DETECTION AND IDENTIFICATION OF RETROVIRAL NUCLEIC ACIDS/RETROVIRUSES IN A SPECIMEN**

APPLICANT(S) FOR DO/EO/US

**WOLFGANG SEIFARTH, CHRISTINE LEIB-MÖSCH & CORINNA BAUST**

**Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:**

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2))
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ Original or facsimile of an oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. concern other document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Disc with the sequence listing

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

007705

INTERNATIONAL APPLICATION NO.

PCT/DE00/01071

ATTORNEY'S DOCKET NO.

SEIFARTH

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):

[X] For filing with EPO or JPO search report (37 C.F.R. 1.492(a)(5))	\$ 890.00	\$890.00
[ ] International preliminary examination fee paid to USPTO (37 C.F.R. 1.492(a)(1))	\$ 710.00	
[ ] No international preliminary examination fee paid to USPTO (37 C.F.R. 1.492(a)(2)) but international search fee paid to USPTO (37 C.F.R. 1.445(a)(2))	\$ 740.00	
[ ] Neither international preliminary examination fee paid to USPTO (37 C.F.R. 1.492(a)(3)) nor international search fee paid to USPTO (37 C.F.R. 1.445(a)(2))	\$1,040.00	
[ ] International preliminary examination fee paid to USPTO (37 C.F.R. 1.492(a)(4)) and all claims satisfied provisions of PCT Articles 33(2)-33(4)	\$ 100.00	

Surcharge of \$130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the  
earliest claimed priority date (37 CFR 1.492(e)).

Claims

Total Claims

Number Field

Rate

Independent Claims

9-20

x \$ 18.00

Multiple dependent claims (if applicable)

1-3

x \$ 84.00

x \$280.00

TOTAL OF ABOVE CALCULATIONS

\$890.00

[X] Applicant claims small entity status pursuant to 37 C.F.R. 1.27.  
Reduction by 1/2 for filing by small entity.

SUBTOTAL

\$445.00

Processing fee of \$130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from  
the earliest claimed priority date 37 CFR 1.492(f).

TOTAL NATIONAL FEE

\$445.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by  
an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

TOTAL FEES ENCLOSED

\$ 0.00

Amount to be refunded

\$445.00

charged

a. [X] A check in the amount of \$445.00 to cover the above fees is enclosed.

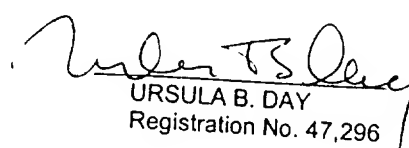
b. [ ] Please charge my Deposit Account No. 06-0502 in the amount of \$ \_\_\_\_\_ to cover the above fees. A  
duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any  
overpayment to Deposit Account No. 06-0502. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR  
1.137(a) or (b) must be filed and granted to restore the application to pending status.

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PCT/PTO 28 MAY 2002  
In der Schanz 10

☎ 06203-61348  
Fax 06203-64196

### Verification Statement

We confirm that the material on the diskette submitted herewith is identical in substance to the Sequence Listing included in the description of the application entitled "Verfahren zum spezifischen Nachweis und zur Identifizierung retroviraler Nukleinsäuren / Retroviren in einem Untersuchungsgut" based on DE 199 21 419.0-44

Schriesheim, April 3, 2000

*Dr. Ulrike Rudolph*

Dr. Ulrike Rudolph

10009705 112401

**PATENT**

**531 Rec'd PCT/F 08 NOV 2001**

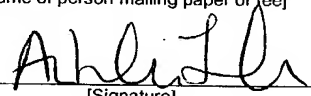
**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Docket No.: SEIFARTH

In re Application of:	)
WOLFGANG SEIFARTH et al.	)
Int. Appl. No: PCT/DE00/01071	)
Int. Filing Date: April 4, 2000	)
For: METHOD FOR THE SPECIFIC	)
DETECTION AND IDENTIFICATION OF	)
RETROVIRAL NUCLEIC	)
ACIDS/RETROVIRUSES IN A SPECIMEN	)

**FIRST PRELIMINARY AMENDMENT**

BOX PCT  
Commissioner for Patents  
Washington, D.C. 20231

Express Mail mailing label number: <b>EV 042503525 US</b>
Date of Deposit: <b>November 8, 2001</b>
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.
<b>ANTONELLA FUSILLO</b>
[Name of person mailing paper or fee]

[Signature]

S I R:

Preliminary to the first Official Action in the above-entitled application, please amend the application as follows:

### Clean Version of Amended Claims

5. A method according to claim 3, characterised in that a mixture of equimolar quantities of both partners of a pair of synthetic oligonucleotides, which together correspond to a preferably 90 bp long section from the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L , is in each case used as immobilised RDBH probes.
7. A use of one or several synthetic oligonucleotide(s) whose nucleotide sequence(s) correspond(s) with the nucleic acid region of a retrovirus-specific reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L or with a section of this nucleic acid region as reverse dot blot hybridisation probe(s) in a method according to claim 3.
8. A use of equimolar quantities of two synthetic oligonucleotides which together, positioned one after the other, correspond to a preferably 90 bp long section from the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L as reverse dot blot hybridisation probe(s) in a method according to claim 3.
9. A diagnosis kit for the specific detection and identification of retroviral nucleic acids/retroviruses in an arbitrary specimen, comprising at least one of the primer mixtures consisting of forward and reverse primers for the PCR according to claim 1 and at least one reverse dot blot hybridisation probe according to claim 7.

**Version Showing the Changes Made**

**IN THE CLAIMS:**

Amend the following claims:

5. A method according to [one of claims 3 or 4] claim 3, characterised in that a mixture of equimolar quantities of both partners of a pair of synthetic oligonucleotides, which together correspond to a preferably 90 bp long section from the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L , is in each case used as immobilised RDBH probes.
7. A use of one or several synthetic oligonucleotide(s) whose nucleotide sequence(s) correspond(s) with the nucleic acid region of a retrovirus-specific reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L or with a section of this nucleic acid region as reverse dot blot hybridisation probe(s) in a method according to [one of claims 3 to 6] claim 3.
8. A use of equimolar quantities of two synthetic oligonucleotides which together, positioned one after the other, correspond to a preferably 90 bp long section from the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L as reverse dot blot hybridisation probe(s) in a method according to [one of claims 3 to 6] claim 3.
9. A diagnosis kit for the specific detection and identification of retroviral nucleic acids/retroviruses in an arbitrary specimen, comprising at least one of the primer mixtures consisting of forward and reverse primers for the PCR according to claim 1 and at least one reverse dot blot hybridisation probe according to claim 7 [or claim 8].

Docket No.: SEIFARTH  
Int. Appl. No.: PCT/DE00/01071

### REMARKS

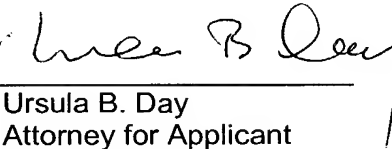
This Amendment is submitted preliminary to the issuance of an Office Action in the present application.

Applicant has amended claims 5, 6-7 and 9 to remove any multiple dependency of the claims.

When the Examiner takes this application up for action, he is requested to take the foregoing into account.

The Commissioner is hereby authorized to charge any additional fee which may be required, or credit any overpayment to Deposit Account No.06-0502.

Respectfully submitted,

By:   
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Attorney for Applicant  
Reg. No. 47,296

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4/PRTS

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531 Rec'd PCT/TT

08 NOV 2001

METHOD FOR THE SPECIFIC DETECTION AND IDENTIFICATION OF

RETROVIRAL NUCLEIC ACIDS/RETROVIRUSES IN A SPECIMEN

Description

**[0001]** The invention relates to a method for the specific detection and identification of retroviral nucleic acids/retroviruses in an arbitrary specimen and a diagnosis kit for implementing this method. It also relates to retrovirus-specific probes for reverse dot blot hybridisation and retrovirus-specific oligonucleotide primer mixtures (MOP) comprising forward and reverse primers for producing amplicates of retrovirus-specific nucleic acids from the specimen.

**[0002]** Exogenous and endogenous retroviruses (HERV) are etiological agents for a multiplicity of tumorigenic diseases in humans and animals. They are involved in the formation of tumours and leukaemias in numerous animal models but also in humans (HTLV-I and II). Others also cause immunodeficiency diseases (HIV). Present research indicates that retroviruses may also play a role as triggers of autoimmune diseases (Kalden and Herrmann, 1993) and neuronal degenerative diseases such as multiple sclerosis (Tuke et al. 1997). Intensive research in the field of endogenous and exogenous retroviruses has so far led to the continual discovery of new retroviral sequences in the human genotype whose expression could possibly be associated with specific diseases. For example, the expression of Gag proteins of the HERV-K family is associated with almost all forms of testicular and ovarian germinomas (Sauter et al. 1995). Antibodies against HERV-K Env protein were detected in human sera (Vogetseder et al. 1993). The HERV-K-IDDM *env* gene which was isolated from patients with type-1 diabetes possibly codes for an endogenous superantigen (Conrad et al. 1997).

**[0003]** Statistically reliable studies using large groups of patients are required to correlate specific diseases with the activity of specific endogenous or exogenous retroviruses. The expenditure in time and money required for this using the known methods of detection according to the prior art is immense.

**[0004]** The increasing use of retroviral vector systems in human gene therapy casts doubts on the safety from undesirable side effects (genome



changes in the target cells, transmission of undesirable viruses). Thus, a certain percentage of undesirable gene sections of endogenous or foreign retroviruses are also co-packaged in the retroviral particles for therapeutic application in packaging cell lines (Co-packaging, Sherwin et al. 1987, Scolnick et al. 1979, Takeuchi et al. 1992). For example, transcripts of certain endogenous retroviruses, such as those present in related form in the genome of packaging cell lines, have been detected in retrovirus-like particles (pseudotypes) of the breast cancer cell line T47D (Seifarth et al. 1995, 1998). The packaging of such undesirable retroviral sequences can lead to the recombination and formation of new retroviruses having modified, possible pathogenic properties. The re-integration of such recombinant retroviruses in the genome of the target cells can lead to insertion mutagenesis and consequently to inactivation of important genes of the cell cycle and possibly to tumorigenesis.

**[0005]** For this reason it is necessary to conduct quality control of the gene vector preparations to be used for gene therapy using a sensitive test system. This could prevent any undesirable retroviral sequences from being transfused. In the event of positive detection, a vector preparation could be subjected to suitable purification (purging) before being administered to the patient. The methods known according to the prior art are not suitable for such an application.

**[0006]** A controversial issue at the present time is the use of animal organs for transplantation in humans (xenotransplantation). For example, as a result of the shortage of suitable donors, heart valves from pigs are being increasingly transplanted in humans. The transplantation of heart, liver and kidney transplants is also planned. However, recent research has shown that within the transplantation framework and the associated immunosuppression by medication, endogenous or exogenous retroviruses so far suppressed in the donor organ can become activated in the recipient. As has already been demonstrated experimentally, these retroviruses of animal origin are pathogenic for certain human cell types (xenotropism) and could thus lead to a serious systemic disease of the organ recipient. In cases where pathogenic infectious virus particles are formed, transmission to uninvolved third parties (epidemic) cannot be excluded. Last but not least, recombinations of retroviruses of animal origin with endogenous human retroviruses could result in new pathogenic virus recombinants with completely new host tropisms.



**[0012]** Developments in the field of molecular biology have led to the development of new methods of detection (hybridisations, PCR) which possess similar sensitivity to serological antigen methods of detection. In this case also, the detection success stands or falls by the availability of virus-specific gene probes (hybridisation) or oligonucleotides (PCR). Since the use of several probes or PCR primers is limited because of non-specific interactions in a reaction formulation, many experiments must be conducted in parallel to detect complex expression patterns.

**[0013]** In view of the circumstances described previously, the problem for the present invention was to provide an efficient and reliable, and at the same time fast method for the multiple detection of endogenous and exogenous retroviruses of human and animal origin.

**[0014]** This problem is solved using a method of the type specified initially which is characterised in that it comprises the following measures:

**[0015]** Isolation of nucleic acids, namely DNA and/or RNA from the specimen,

- carrying out a PCR using the isolated DNA or an RT-PCR using the isolated RNA using one or both the primer mixtures MOP-ABD and MOP-C described hereinafter, each consisting of forward primers and reverse primers, whose forward and reverse primers are degenerated oligonucleotides having the nucleotide sequences reproduced in the sequence protocols SEQ ID NO.1 to NO.4 according to the IUPAC nomenclature and a so-called "head" at the 5'-end of these nucleotide sequences, whereby the forward primers of the MOP-ABD mixture exhibit the nucleotide sequences in accordance with SEQ ID NO.1,





particles are to be studied, polyadenylate messenger RNA (mRNA) free from genomic DNA must be isolated. If mRNA is used as the starting material, this mRNA must be transcribed into complementary DNA (cDNA) *in vitro* by means of reverse transcriptase and can then be used as matrices for the following PCR. This combination of reverse transcription and PCR is generally described as RT-PCR.

**[0021]** The isolated nucleic acids are then subjected to a one-step PCR using primer mixtures according to the invention (MOP-ABD, MOP-C) consisting of retrovirus-specific, degenerated oligonucleotides corresponding to the highly conserved regions within the reverse transcriptase gene (RT gene) of all known human retroviruses (MOP, Shih et al. 1989, Donehower et al. 1990). In this PCR all retrovirus-specific 'reverse transcriptase' homologous sequence sections contained in the specimen are amplified. As a result an amplificate mixture of short retroviral DNA fragments is obtained whose composition reflects the frequency of all retroviral nucleotide sequences to be detected in the specimen. The amplificates are either labelled during the PCR reaction or after this reaction, preferably radioactively, but equally well non-radioactively according to choice (e.g. with biotin or digoxigenin). These labelled amplificates are then used as probes in a hybridisation method (RDBH method) using filter membranes or bio-chips with applied retrovirus-specific oligonucleotides as probes.

**[0022]** The head or extension sequence of the primer oligonucleotides according to the invention consisting of clamp and interface sequence first has the positive effect that it favourably influences the primer matrix binding kinetics so that the PCR products formed in the first PCR cycle are amplified substantially more efficiently in the following cycles. This has the advantage that retroviral matrices are then amplified themselves or can be amplified if the exactly matching primer is not present in the primer mixture. The interface also has the advantage that it facilitates cloning if necessary.

**[0023]** It basically holds that the length of the head or extension sequence should not exceed half the length of the complete primer nucleotide sequence.



**[0024]** An important component of the method according to the invention are the RDBH probes used for the reverse dot blot hybridisation (RDBH) which comprise specific quantities of synthetically produced, exactly defined nucleic acid sequences from the reverse transcriptase gene of those retroviruses already characterised against which the specimen is to be tested. These retrovirus-specific RDBH probes are applied to defined fields (dots) of the RDBH support whereby this can be conventionally used filter membranes having dimensions of several centimetres and also so-called bio-chips having dimensions of a few millimetres (micro-array technology). In the case of filter membranes the probes are preferably linked to these supports covalently by UV crosslinking (e.g. using the commercially available UV radiation source 'Stratalinker<sup>TM</sup>', Stratagene).

**[0025]** In the case of DNA chips, oligonucleotide probes are synthesised in situ and fixed in precisely defined positions on a solid support using photolithographic methods (similar to engraving) (e.g. by using perforated masks specific areas of the chip are illuminated to activate photosensitive chemical groups). The support, preferably a glass or nylon surface of approximately 1 cm<sup>2</sup> forms the hybridisation unit. Each hybridisation unit can contain a very large number of different oligonucleotide probes (up to 400,000). As a result many thousand different sequences can be analysed simultaneously. For each probe all sequence alternatives are presented on the chip and a single one must be recognised by the specimen to be tested. The hybridisation of probe and target sequence (in the specimen) is detected by measuring the amplificate marker intensity. The intensity is proportional to the extent of the hybridisation between probe and target sequence. Each target sequence is identified according to its hybridisation position on the chip. The DNA chip technique was developed by FODOR et al. (Science 251, 767-773, 1991) and is known in the prior art (see V. Oeding et al. 1999, *HYGIENE UND MIKROBIOLOGIE* 1/99, pp. 55-57 and G. Ramsay 1998, *NATURE BIOTECHNOLOGIE*, Vol. 16, 1998, pp. 40-44).

**[0026]** Hybridisation should take place under highly stringent conditions matched to the length of the probes and the hybridised supports (filter membranes or chips) should then be washed under highly stringent conditions. In cases where the PCR amplificates are radioactively labelled, the identity of



the detectable retroviruses can then be identified after exposing the filter membranes on an x-ray film using the signal pattern of the autoradiograms.

**[0027]** The method according to the invention can be used for the multiple detection and identification of all human and/or animal-specific retroviral nucleic acids/retroviruses known so far in cell cultures, cell culture residues or body samples or other specimens of biological origin. The only prerequisite is that certain genome sections of the retroviruses to be detected, namely the conserved sequence regions of the reverse transcriptase are known with respect to their DNA nucleotide sequence. This prerequisite is satisfied since the corresponding nucleotide sequences of exogenous and endogenous human retroviruses are generally accessible as gene bank data.

**[0028]** The method according to the invention for the first time opens up the possibility of providing a universal retrovirus detection system with which the entire spectrum (= expression pattern) of all active endogenous and exogenous retroviral nucleotide sequences in the specimen (body sample) can be determined in a single experiment. In particular, this detection system can also be used to carry out statistically reliable studies using arbitrarily large groups of patients and if appropriate correlations existing between specific diseases and the activity of specific endogenous or exogenous retroviruses can be established therefrom. In the event of a proven correlation between a specific retroviral expression pattern and a specific disease, this test system can also be used for the early identification or to assess the personal genetic risk for such a disease.

**[0029]** Another very decisive advantage of the method is that the sequences of the PCR primers (MOP) according to the invention, i.e., the degenerated oligonucleotides of the primer mixtures used to amplify the retrovirus-specific RT gene sections to be identified in the specimen do not overlap with the sequences of the synthetic virus-specific oligomers used as dot blot probes. The PCR primer sequences contribute approximately half the final amplificate length. If these sequence sections were also contained in the dot blot probes (cf. Herrmann and Kalden, 1994), this would lead to considerable restrictions in the prediction capability of the test since the amplicates would then hybridise to a certain extent with all dot blot probes on the filter membrane. It is an advantage of the method according to the invention that this undesirable



effect is impeded by using synthetic, precisely defined, homogenous oligonucleotide preparations for both the forward and reverse primers and for the RDBH probes.

**[0030]** The nucleotide sequences of all exogenous and endogenous retroviruses characterised so far (HERV) are published in gene banks. From these suitable nucleotide sequences can be derived for the synthesis of virus-specific oligomers as dot blot probes. In principle, corresponding oligomers of all these sequences can be dotted onto a single filter membrane. Consequently, it is possible to test a specimen for the entire spectrum of retroviruses known so far in a single experiment. Compared with the prior art whereby its own diagnostic test, especially a serological test using a special antibody, must be carried out to identify each putative virus in a sample, the method according to the invention thus represents significant progress.

**[0031]** As a result of the known higher sensitivity of PCR and the possibility of repeated amplification of PCR products, the method according to the invention also attains a detection limit which is barely achieved by any other test system.

**[0032]** In a preferred embodiment of the method according to the invention the "head" or extension section of the primer nucleotides according to the invention consists of the nucleotide sequence GAAGGATCC whereby the nucleotide series GAA is a so-called 'clamp' and the nucleotide series GGATCC represents the interface for the restriction enzyme BamHI. The head or extension sequence has proved very good in practice. In principle, however, the head sequence can also consist of any arbitrary nucleotide sequence provided that the primer annealing kinetics is not negatively influenced.

**[0033]** The nucleotide sequences of the synthetic oligonucleotide of the RDBH probes are preferably selected so that they correspond to the retroviral nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L or to a section of this region i.e., they match and/or (experimentally) can hybridise with it.

**[0034]** Since the efficiency of an oligonucleotide synthesis decreases with the length of the oligonucleotide to be synthesised, a variant of the method





according to the invention is provided in which a mixture of equimolar quantities of two comparatively short-chained synthetic oligonucleotides is used as immobilised RDBH probes in each case (i.e. for each probe or each dot). These correspond together or one after the other to a longer, preferably approximately 90 base pairs (bp) long, section of the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L.

**[0035]** In an embodiment that has proved very useful in practice, these two short-chain oligonucleotides are approximately the same size or the same length and preferably comprise approximately 45 base pairs.

**[0036]** The invention is explained subsequently in greater detail with reference to examples of embodiment and relevant drawings and tables.

**[0037]** The abbreviations used are as follows:  
 BaEV = baboon endogenous retrovirus;  
 ERV = endogenous retrovirus;  
 ERV9 = endogenous retrovirus type 9.  
 GaLV = gibbon ape leukaemia virus;  
 HERV = human endogenous retrovirus;  
 HIV = human immunodeficiency virus;  
 HML = human mouse mammary tumour virus-like sequence;  
 HPLC = High Performance Liquid Chromatography  
 HRV5 = human (exogenous) retrovirus type 5;  
 HTLV-1 = human adult T-cell leukaemia virus type 1;  
 LINE = long, disperse (scattered) DNA sequence element;  
 MMTV = mouse mammary tumour virus;  
 MoMuLV = Moloney mouse leukaemia virus;  
 MOP = Primer mixture of degenerated oligonucleotides comprising forward and reverse primers  
 MPMV = Mason Pfizer monkey virus;  
 PCR = polymerase chain reaction;  
 PERV = porcine endogenous retrovirus;  
 PBMC = peripheral blood mononuclear cells;  
 RDBH = reverse dot blot hybridisation;  
 RT = reverse transcriptase.

**[0038]** Table 1: Retrovirus-type ABD and type C specific primer mixtures MOP-ABD and MOP-C according to the invention which each contain forward and reverse primers and comprise degenerated oligonucleotides. The standardised single-letter abbreviation code of the IUPAC nomenclature has been used to describe the degenerated oligonucleotide sequences (see *European Journal of Biochemistry* 150: 15, 1985). Both the forward primer and the reverse primers are shown in the 5'-3' direction following the IUPAC conventions and relative to the DNA strand. The degree of degeneration, in other words the number of different specific embodiments of this primer that can be obtained by synthesis, is given in each case in the form of the theoretically calculated number of different oligonucleotides.

**[0039]** Table 2: Immobilised synthetic retrovirus-specific oligonucleotide probes for producing dot blot membranes. A mixture of equimolar quantities of both partners of an oligonucleotide pair which corresponds to a 90 bp long section of a retrovirus-specific reverse transcriptase was prepared for each spot or dot. In each case 100 picomole of these mixtures was applied to the membrane in the configuration corresponding to the code shown. A dilution series of human genomic DNA (8E-8H) and oligonucleotide primer mixtures (8I-8L) was applied to the filter for internal standardisation of the hybridisation and autoradiography. For each oligonucleotide sequence used on the filter the gene bank access number and the first author are given where available.

**[0040]** Table 3: Classification of retrovirus-specific oligonucleotide dot blot probes: From 61 representative members of all known human exogenous and endogenous retroviruses the nucleotide sequence of the appropriate reverse transcriptase gene in the region between the highly conserved domains V L P Q G and Y M/V D D I/V/L L was used to synthesise dot blot probes (Shih et al. 1989, Donehower et al. 1990). In the experiment shown here 21 retroviral nucleotide sequences from type ABD (HERV-K superfamily), 19 retroviral nucleotide sequences from type C, 1 retroviral nucleotide sequence from type D and 7 nucleotide sequences related to the human foamy virus were used. Also tested were a human LINE-1 sequence (3L) and 6 exogenous human retroviruses (6E-6J), as well as five probes which correspond to a mammal C type retrovirus and a probe which corresponds to a mammal B type retrovirus (7E-7J).

**[0041]** Asterisks indicate nucleotide sequences related to HERV transcripts which were found in patients with multiple sclerosis and patients with systemic lupus erythematosus.

**[0042]** FIG. 1 Localisation of conserved amino acid sections in the amino-terminal gene regions of the reverse transcriptase of retroviruses and retrotransposons. The core homology regions V L P Q G and Y M/V D D I/V/L L were used to derive and prepare the degenerated oligonucleotides of the primer mixtures according to the invention (MOP-ABD or MOP-C).

**[0043]** FIG. 2. Schematic of the RT-PCR/RDBH method according to the invention.

**[0044]** FIG. 3. HERV expression pattern in human PBMNCs of a healthy blood donor.

**[0045]** The reverse dot blot hybridisation (RBDH) was carried out under standard conditions using DNA fragments which had been amplified using the primer mixtures of degenerated oligonucleotides according to the invention, namely MOP-ABD (Table A) or MOP-C (Table B) or the combination MOP-ABD/MOP-C (Table C).

**[0046]** FIG. 4. HERV expression in human PBMNCs after adding a cloned DNA fragment that contains a PERV RT gene. Less than 10 copies of a porcine endogenous retrovirus (PERV) type A DNA (Patience et al. 1997) could be detected and identified under standardised test conditions (Table A, filter code 7F). Under the stringent conditions used no cross hybridisation of HERVs with porcine-specific amplification products obtained from a porcine DNA matrix were observed (Table B).

**[0047]** Example 1: RNA preparation

**[0048]** Total RNA was extracted from peripheral blood mononuclear cells of a healthy blood donor using the guanidine isothiocyanate/caesium chloride (GIT/CsCl) ultracentrifugation protocol proposed by Sambrook et al. (1989) and dissolved in distilled water treated with diethylpyrocarbonate (DEPC). The



mRNA was then enriched using conventional methods e.g. using the commercially available enrichment kit 'Dynabeads™ paramagnetic particles' according to the manufacturer's instructions (Dyna, Hamburg, Germany). The nucleic acid concentration was determined by means of spectrometry at 260 nm. In order to check for any contamination with genomic DNA, 50 ng of each mRNA preparation was used directly, i.e., without first having undergone a reverse transcription, in a polymerase chain reaction (PCR) using the primer mixtures of degenerated oligonucleotides (MOP) according to the invention. Only those preparations which showed no DNA traces were used for the actual PCR. Those preparation formulations for which some DNA contamination could be detected were treated with 100 units/μg RNase-free DNase (Roche Diagnostics, Mannheim Germany) in 100 mM pH 5.0 sodium acetate, 5 mM MgSO<sub>4</sub>, until the control PCR yielded a negative result.

**[0049]** Example 2: Preparation of MOP-ABD and MOP-C primer mixtures according to the invention for PCR

**[0050]** Table 1 shows preferred MOP-ABD and MOP-C primer mixtures using the IUPAC nomenclature familiar and commonly used in specialist circles. Each of the primer mixtures contains a plurality of different forward and reverse primers. The forward primers of the MOP-ABD primer mixture exhibit the general nucleotide sequence GAAGGATCCARAGTNYTDYCHCMRGGH which comprises 3456 degenerations, i.e. 3456 different specific nucleotide sequences. The reverse primers of the MOP-ABD primer mixture exhibit the nucleotide sequence GAAGGATCCNWDDMKDTYATCMAYRWA which comprises 27648 degenerations, i.e. 27648 different specific nucleotide sequences. The forward primers of the MOP-C primer mixture are characterised by the general nucleotide sequence GAAGGATCCTKKAMMSKVYTRCYHCARGGG, which comprises 3072 degenerations, i.e. 3072 different specific nucleotide sequences and the reverse primers of the MOP-C primer mixture exhibit the nucleotide sequence GAAGGATCCMDVHDRBMDKYMAYVYAHKKA which comprises 8192 degenerations, i.e. 8192 different specific nucleotide sequences.

**[0051]** These primer nucleotide sequences correspond to the highly conserved core homology regions V L P Q G and Y M/V D D I/V/L L within the reverse transcriptase (RT) gene of all known endogenous and exogenous



retroviruses (see Fig. 1 and the publications of Xiong and Eickbush 1990, Shih et al. 1989 and Donehower et al. 1990). The beginning of the primer nucleotide sequence at the 5' end of the appropriate retrovirus-specific core-homology region, called the "head", namely the nucleotide series GAAGGATCC is an extension sequence which consists of the so-called "clamp" sequence GAA and the *Bam*HI restriction site GGATCC.

**[0052]** Instead of the "clamp" sequence described here and the *Bam*HI restriction site described here, another "clamp" sequence and/or another interface for a restriction enzyme can also be used to produce the "head" or extension sequence of the appropriate primer. Basically however the length of this "head" or extension sequence should not be more than half the total primer length.

**[0053]** The MOP-ABD primer mixture allows the separate amplification of types A, B and D retrovirus and the MOP-C primer mixture allows the separate amplification of type C retrovirus. Both primer mixtures can easily be combined and thus make it possible to amplify all retrovirus types (A, B, C, and D).

**[0054]** Example 3: preparation of probes for reverse dot blot hybridisation (RDBH)

**[0055]** Amino acid sequence comparisons have shown that the coding genes of all retrovirus for the reverse transcriptase and most retroelements exhibit highly conserved core homology regions (Poch et al. 1989, Shih et al. 1989, McClure 1993, Donehower et al. 1990, Xiong and Eickbush 1990). Two of the most conserved amino acid sequence sections are the amino acid motifs V L P Q G and Y Y/M D D I/V/L L (Fig. 1). The sequence region between these motifs comprises approximately 90 base pairs (i.e. is approximately 90 bp long) and exhibits a significantly lower homology within the various retrovirus families. This region was used to prepare retrovirus-specific probes for RDBH.

**[0056]** Here the following procedure was adopted: first, generally accessible nucleotide sequence data banks were searched for nucleotide sequences related to the nucleotide sequence of the reverse transcriptase (RT). Sequences of exogenous and endogenous retroviruses were classified according to the valid nomenclature and subclassified into subclasses in terms

of their RT homology (data not shown here). Some as yet unpublished HERV sequence data were kindly made available by Martin Herrmann (1998) and some were characterised by ourselves. Representative members were selected from all known retrovirus families (Table 3) and in each case an approximately 90 bp long fragment was isolated from their respective RT gene, in each case in the region between the highly conserved RT motifs V L P Q G and Y M/V D D I/V/L L, and was used as a template for synthesising the corresponding RDBH probes. Since the efficiency of an oligonucleotide synthesis decreases with the length of the oligonucleotide to be synthesised, instead of a 90 pb long oligonucleotide (90-mer) two 45 bp long oligonucleotides (45-mers) were synthesised in each case and used as a pair. Each dot (spot) of the dot blot prepared as described here corresponds to an equimolar mixture of equal proportions of one pair of 45-mers from the group of pairs listed in Table 2.

**[0057]** Example 4: preparation of reverse dot blot membranes for RDBH

**[0058]** Retrovirus-specific oligonucleotides which correspond to a 90 bp long fragment of the highly conserved domains of the RT gene were synthesised and purified by HPLC. For each retroviral nucleotide sequence to be tested equimolar quantities of both partners of a pair of 45-mer oligonucleotides prepared as in example 3 were mixed together and 100 picomole of this pair mixture was dissolved in 5x SSC (1x SSC = 0.15M NaCl plus 0.015 M sodium citrate) and then dropped manually or by machine onto a commercially available filter membrane (for example, a ZETAprbe™ GT blotting membrane supplied by BioRad, Hercules CA USA) using a commercially available dot blot apparatus (for example, Minifold I dot blotter SRC96D made by Schleicher & Schuell, Dassel, Germany). The filters were equilibrated in 2x SSC, the oligonucleotides were irreversibly immobilised, preferably by means of UV cross linking (for example, using the commercially available UV emitter Stratalinker™ supplied by Stratagene, La Jolla, CA USA) and the filters were then dried in air.

**[0059]** After the amplificate DNA has been hybridised to the RDBH probes covalently linked to the membranes, bound amplificate DNA can be re-dissolved from the dot blot membrane by alkaline denaturation and if necessary re-amplified to achieve sufficient quantities of double-strand DNA, e.g. for cloning and subsequent sequence analysis of the amplificates concerned.



**[0060]** Example 5: reverse transcription and polymerase chain reaction (RT-PCR)

**[0061]** From each test formulation 500 ng of DNA-free mRNA were reverse transcribed at 37°C for 1 hour in 50 µl of a solution of 20 mM pH 8.4 tris/HCl, 10 mM dithiothreitol (DTT), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.5 mM of each desoxynucleoside triphosphate (dNTP), 10 units of RNasin (Promega) 30 pmol Random hexamer oligonucleotides (Promega) and 20 units of MLV reverse transcriptase (GIBCO-BRL).

**[0062]** The formulations were then denatured, for example by heat treatment at 95°C for 5 min and stored at -20°C before further usage.

**[0063]** For the MOP-PCRs according to the invention (with MOP-ABD and/or MOP-C) in each case a volume of one twentieth (1/20) of the cDNA reaction was amplified in 50 µl of a solution of 10 mM pH 8.3 tris/HCl, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.001 % gelatine, 50 pmoles of the relevant primer mixture(s) of degenerated oligonucleotides according to the invention, 0.25 mM of each desoxynucleoside triphosphate and 1.25 units Taq polymerase (GIBCO-BRL). The test formulations were prepared on ice and coated with 50 µl of mineral oil (Sigma). Amplification was carried out in a commercially available DNA thermal cycler (for example supplied by Perkin Elmer Cetus) using the "Hot-Start method" familiar to the specialist whereby 30 cycles were run through, each having the following parameters: 30 sec at 94°C, 4 min at 50°C and 1 min at 72°C. Finally an extension step was carried out at 72°C for 7 min. The annealing time, i.e. the time taken for double strand formation, was 4 minutes in order to ensure that the vast majority of the primers (degenerated oligonucleotides) contained in the primer mixture according to the invention find the matrices homologous to them. The extension sequence of the primer according to the invention has a stabilising effect on the primer matrix binding kinetics so that the PCR products formed in the first PCR cycle are amplified significantly more efficiently in the following cycles. There is thus the advantage that retroviral matrices are (can be) then amplified themselves if the exactly matching primer is not present in the primer mixture according to the invention. In addition, it is possible to achieve fast cloning of the amplification products e.g. for a sequence examination or to characterise new RT-related nucleotide sequences.

**[0064]** The reaction conditions for the PCR were optimised with respect to the amount of primer, the annealing time (double strand formation time) and the annealing temperature (double strand formation temperature) in order to achieve an optimum product yield.

**[0065]** In order to detect product contamination from previous PCR experiments and any traces of genomic DNA contaminations in the solutions used, a control reaction was carried out in which the matrices were omitted. The amplification products were separated electrophoretically on preparative 2.5 % TBE agarose gels and stained with ethidium bromide. Bands of between 100 and 150 bp which corresponded to the amplified retroviral RT nucleotide sequences were cut from the gel and cleaned using a commercially available cleaning set (for example, the GENECLAN II kit from BIO 101 Inc., Vista CA USA). For the RDBH approximately 50 ng of the cleaned fragment was labelled with [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mmol). The labelling was carried out using a Megaprime DNA labelling kit (Amersham Pharmacia Biotech, England) but can also be carried out equally well using other common labelling methods.

**[0066]** Example 6: reverse dot blot hybridisation (RDBH)

**[0067]** The RDBH method was used both to detect and to identify the amplified products. This RDBH method can strictly discriminate (distinguish) PCR products so that any wrongly amplified nucleotide sequences which are not related to nucleotide sequences of retroviral RT genes are of no importance. The high stringency of the RDBH is achieved by using synthetic HERV-specific oligonucleotides according to the invention which are applied as RDBH probes onto the dot blot filter membrane. The important advantage of these RDBH probe oligonucleotides according to the invention is that they contain none of the nucleotide sequences which are exhibited by the degenerated oligonucleotides of the MOP-ABD and MOP-C PRC primer mixtures according to the invention (see, for example, Table 1) and thus differ fundamentally from these PCR primer oligonucleotides. This fundamental difference between the RDBH probe oligonucleotides and the PCR primer oligonucleotides ensures that a hybridisation between an RDBH probe and a PCR amplificate only takes place if the nucleotide sequence between the two primers corresponds to the relevant RDBH probe oligonucleotide, i.e. if this



nucleotide sequence is identical to the relevant RT nucleotide sequence section or only differs in a few nucleotides ( $n = 3$ ). In the ideal case the hybridising DNA sequences should be completely identical. In practice, under the given hybridisation conditions differences of two to three nucleotides are tolerable. Consequently, under highly stringent conditions even closely related retroviral nucleotide sequences can be distinguished from one another and be uniquely identified.

**[0068]** In order to avoid cross hybridisations, the optimum stringency conditions for the RDBH were determined by varying hybridisation temperature, washing temperature and salt concentrations. Pre-hybridisation of the reverse D blot filters was carried out in heat-sealed plastic pockets in 0.25 M pH 7.2  $\text{Na}_2\text{HPO}_4$ , 7 % sodium dodecylsulphate (SDS), 1 mM EDTA at 50°C for at least 3 hours. For the actual hybridisation these solutions were mixed with  $5 \times 10^5$  CpM of the labelled PCR amplificate per ml hybridisation volume and incubated for 16 hours under the same conditions. The membranes were then washed twice in 40 mM pH 7.2  $\text{Na}_2\text{HPO}_4$ , 5 % SDS, 1 mM EDTA and twice in 40 mM  $\text{Na}_2\text{HPO}_4$  pH 7.2, 1 % SDS, 1 mM EDTA (for approximately 30 min in each case). The reaction was studied and evaluated by autoradiography.

**[0069]** Example 7: Analysis of the HERV transcription pattern in human PBMNCs using the PCR/RDBH method according to the invention

**[0070]** First, using the method shown in Fig. 2, total RNA was extracted from human PBMNCs as in Example 1 using common isolation techniques. This total RNA was first subjected to an RT-PCR/RDBH using the MOP-ABD primer mixture according to the invention. Here almost exclusively type B-related HERVs, i.e. members of the HERV-K superfamily were detected (Fig. 3A). The most transcripts originated from members of the HERV-K subgroups HML-2, -3, -4 and -6. Also found were signals from HERV-KC4 related elements (8A, 8B) and from another HERV-K related nucleotide sequence which could not be assigned to any of the HML subgroups (5F). The observed expression pattern agrees with the studies already published which established a differentiated expression of HML elements in human tissue in the result (Medstrand et al. 1993, Andersson et al. 1996). Also found were small quantities of the element HERV-L related to the human foamy virus whereby

the high specificity of the MOP-ABD primer mixture for type ABD related elements is shown.

**[0071]** At the same time as this test the total RNA from PBMNCs was subjected to an RT-PCR/RDBH using the MOP-C primer mixture according to the invention. Unlike the MOP-ABD primer mixture, the MOP-C primer mixture is not only suitable for priming type-C related nucleotide sequences but also amplifies HERV-K related elements of the HML-2, HML-4 and HML-6 subgroups. A strong expression of HERV-E4-1 related elements (2H and 2I), human foamy virus related HERV-L elements (1E to 1K) and ERV9 related HERVs (4E to 4G and 4I) could be detected. Although the same quantities of radioactively labelled PCR-amplificates were used in all RDBH reactions, the genomic DNA probes present on the membranes (8E to 8H) after hybridisation using MOP-C produced PCR amplificates yielded significantly stronger signals than after hybridisation using MOP-ABD produced PCR amplificates. These findings indicate that the human genome contains significantly more copies of type C related HERV elements than type B related HERV elements.

**[0072]** In order to detect all retroviral nucleotide sequences in a single experiment the MOP-ABD and MOP-C primer mixtures were used in a combination of equimolar quantities in a PCR/RDBH method according to the invention. This experiment resulted in a predominant amplification of type C related nucleotide sequences whereas the ABD type sequences remained underrepresented (data not given here). For this reason separate PCR methods were carried out using first MOP-ABD primer mixtures and second MOP-C primer mixtures and the purified amplification products of both methods were combined in equal quantities. The RDBH was then carried out using this combination of amplification products. The signal pattern shown in Fig. 3C was obtained which corresponds to the theoretical combination of the signal pattern of the RDBH method using MOP-ABD amplificates as in Fig. 3A on the one hand and using MOP-C amplificates as in Fig. 3B on the other hand. This finding shows that the PCR/RDBH method according to the invention is overwhelmingly well suited especially as a qualitative method of detection.

**[0073]** Example 8: Proof of the sensitivity of the PCR/RDBH method according to the invention

**[0074]** In order to check the sensitivity of the PCR/RDBH method according to the invention with reference to the desired practical application in routine diagnostics e.g. to detect or eliminate any interspecies transmission of PERV with xenotransplants, dilution series experiments were carried out using cDNA from human PBMNCs and decreasing concentrations of a cloned DNA fragment that contains a PERV RT-coding region (Takeuchi et al. 1998). Under standardised test conditions even such a small quantity as 10 copies of PERV DNA could be detected in cDNA obtained from 25 ng human PBMNC mRNA (see Fig. 4A, Filter code 7F).

**[0075]** No cross hybridisations between human specific amplicates and PERV specific RDBH probes were observed (see Fig. 3C, filter code 7F). Even when the PCR/RDBH method was carried out using pure porcine DNA as PCR matrix (Fig. 4B), no cross hybridisations could be detected between the porcine amplicates and the human endogenous or exogenous retroviral nucleotide sequences. These results are a very strong indication of the very high interspecies specificity of the PCR/RDBH method according to the invention.

**[0076]** The results obtained in the test described here, namely the weak signal obtained with the murine type C retrovirus specific probe (7 I) surprisingly indicate that the porcine genome DNA also contains PERVs homologous to MoMuLV. Further results of this test, namely the observation of a weak signal with human DNA probes (8E, 8F) also surprisingly indicate that even the human genome possibly contains PERV related nucleotide sequences which have no counterpart on the dot blot membrane used and consequently are probably still uncharacterised. These findings clarify the extraordinary advantage of the PCR/RDBH method according to the invention, namely that it is possible to find, isolate and clone as yet unknown DNA fragments.

Table 1

primer	nucleotide sequence	degeneration
MOP-ABD	forward- <u>GAAGGATCC</u> ARAGTNYTDYCHCMRGGH	3456
	reverse- <u>GAAGGATCC</u> NWDDMKDTYATCMAYRWA	27648
MOP-C	forward- <u>GAAGGATCC</u> CTKKAMMSKVYTRCYHCARGGG	3072
	reverse - <u>GAAGGATCC</u> MDVHDBMDKYMAYVYAHKKA	8192

Table 2

Code	Source	Probe	Oligonucleotide sequence (5' ->3')
1A	U35102, Medstrand et al.1993	HML-1	ATGCTAAATAGCCCAACTGTTTGTAACTTATGTCAGAAAG ATGTTAAATAGCCCAACTATTTGTCAAACCTATGTTGGGAAA
1B	S77579, Levebvre et al. 1995	SEQ29	ATGTTAAATAGCCCAACTATTTGTCAAACCTATGTTGGGAAA ATTAAGCCAGTTAGAGAACAGTTTTAAAAATGTTATAGTATT
1E	G895836, Cordonnier et al. 1995	HERV-L	TATATCAACTCTCCGGCTTTGTGTCATAATCTTATTCAGAGT CTTGATCACTTTTCACTGCCACAAGATATCACACTGGTCCAT
1F	Herrmann 1998	SEQ39	GTATATCAACTCTCCAGCTTTGTGTCATCATCTTATTCAGAG CCTTGATCACTTTTCACTTCTGCAAGATATCATGCTGGTCCA
1G	Herrmann 1998	SEQ40	TTAATCAACTCTCTAGCTTTGTATCATAATCTTATTCGGAGA CCTGATCGCTTTTTCGCTTCCGCAAGATATCACACTGGGTTT
1H	Herrmann 1998	SEQ45	TATATCAGTTATCTGGCTTTGTGACGTAATCTTATTTGGAGA CTAGATAACTTTTCACTTCCACAAGATATCACACTGGTCCAC
1I	Herrmann 1998	SEQ48	TATATCAACTCTCCAGCTTGTGTTATAATTTTATTCAGAGAG TTGATCACTTTTTGCTTCCACAAGATATCACACTGATTGCCT
1J	Herrmann 1998	SEQ51	TGTATCAACTCTCTGGCTTTGTGTCATAATCTTACCTGGAGA CTTGATCGCTTTTTGCTTCCACNAGATATCACACTGGTCCAT
1K	Herrmann 1998	SEQ58	TATATCAACTCTCCAGTTTTGTGTCATAGTCTTAGTCAGAGA CTTGATCACTTTTTGCTTCCATAAGACATCACACTGGCCCAT
2A	M14123 Ono et al. 1986	HERV-K10	ATGCTTAATAGTCCAACCTATTTGTCAGACTTTTGTAGGTCTGA CTTCAACCAGTGAGAGAAAAGTTTTTCAGACTGTTATATTATT
2B	U87592 Zsiros et al.. 1998	SEQ U87592	ATGCTTAATAGTCCAACCTATTTGTCAGACTTTTGTAGGTCTGA CTTCAACCAGTTAGAGAAAAGTTTTTCAGACTGTTATATCATT
2E	U12970 Haltmeier et al. 1995	pCRTK1	TTTAAAACTCCCCTACCCTTTTTGGGGAAGCCCTCCAACA CTTATACCATTCTGAGCCAGTAACCCTCACTGCACTCTTCTC G
2F	U12969 Haltmeier et al. 1995	pCRTK6	TTTAAAAATTCCGCCACCCTTTTTGGGGAAGCCCTCCAACA CTTCTACCATTCTGAGCCAGTCCCCTTAAGTGAAGTCTTCT
2H	M10976 Repaske et al. 1985	HERVE41	TTCAAGAACTCCCCCACCATCTTTGGGGAGGCGTTGGCTCG CCTCCAGAAGTTTCCCACCAGAGACCTAGGCTGCGTGTTGC C
2I	Herrmann 1998	SEQ32	TTCAAGAACTCCCTTACTATCTTCGGGGAGGCTCTGACTTG TTGCAAAAGTTTCCTGCTAAAGACCTAGGCTATGTCTTGCTC G

2J	AF026252, Lindeskog et al. 1998	HERV-H	TTCACAGACAGCCCCCATTACTTCAGTCAAGCCCAAATTTCT TTATCTGTTACCTATCTCCGCATAATTCTCATAAAAAACACAC
2K	Herrmann 1998	SEQ61	TTCAGAGACAGCCCCCATTACTTTAGTCAAGCTCTTTCTCAT CTACTTTCTTTCCATCCATCTGTTTCTCACCTTATTCAATAC
2L	Herrmann 1998	SEQ66	TTCAGAGACAGCCCCCATTACTTTAGTCAAGCTCTTTCTCAT CTACTTTCTTTCCATCCATCTGTTTCTCACCTTATTCAATATA
3A	U35236, Medstrand et al. 1993	HML-3	ATGTTAAACAGTCCAACAATTTGCCAGACTTATATGGGCAAG TTGAACCTACTCTTAAAAAATTTTCACAGTGTTACATTATTCA
3B	S66676, Kalden und Herrmann, 1993	HERV1 SLE	ATGATAAACAGTCCAACAATTTGCCAGGCATATGTAGGGCA AAWTGAACCTACYTGTAaaaaaATTTTCAGTGTTACATTATTC
3C	S77583 Lefebvre et al. 1995	RT244	ATGTTAAACAGTCCCACAATTTGCCAGTCATATGTGGGCCA ATTGAACCTACTCCTAAAAAATTTTCACAGTGTTACATTATTC
3D	Herrmann 1998	SEQ26	CGTGTTAAACAGTCCGACTATTTGCCAGAAGTATGTGGGGG CAATTGAATCTACTCGTAAAAAATTTTCACAGTGTTACATTAT
3E	U27240 Seifarth et al. 1995	ERV-FRD	TTCAGAGATAGTCCCCATTTGTTTGGGCAAGCCTTGGCTAG TTTGCAGGACCTAAGTCTTTATATGGAAGGGCATCTCCTAC
3F	Herrmann 1998	SEQ46	TTCAGAGATAGTCCCCATTTGTTTGGCAAGCCTTGGCTAGA TGCAGGACCTAAGTCTTTATATGGGAGGGCATCTCCTACAG A
3H	M92067 Maeda and Kim 1990	HERV-I	TTCATGGATTACCCCAACCTTTTTGGTCAAATTTTAGAACAA CTAGACAAAGTTTCTGTTCCAAAAACAATTATGCCTGCTTCAA
3I	U27241 Seifarth et al. 1995	HERV-IP (T47D)	TTCACAGACTCCCCTAATATTTTTGGTGAAATTTTAGAACAA ATTAGAAAAAGTTTTCAATTCCAGAACAAATATGCCTTCTCCA
3J	Herrmann 1998	SEQ65	AGACTCCCCTAATCTTTTTGGCCAAATTTTAGAACAAAGTGT AAAAAGTGGTCATCCCAAAGCAAATATGCTTGCTCTAGTACA
3L	McMillan and Singer 1993, M80343	LINE-I	TGCCCTCTCTCACCCTCCTATTCAACATAGTGTTGGAAGTT GCCAGGGCAATTAGGCAGGAGAAGGAAATAAAGGGTATTC
4A	Herrmann 1998	SEQ34	ATGTTAAATGGTCCCACAATTTGCCAGACATATGTGGGGCA CTTGAACCTACTCATAAAAAAATTTTCAGTGTTACATTATTCAC
4B	Herrmann 1998	SEQ42	ATGTTAACTGTCCAACAATTTGTCAGACTTATGTAGAACAA ATTGAACCTACTCATAAAAAAATTTTCAGTGTTATATTATTCAT
4C	Herrmann 1998	SEQ43	ATGTTAAACAGTCCAACAATTTGCCAGATGTACGTGGTGCA AATTGAACCTACTTGTAaaaaaATTTTGGTGTTACATTNTTCN
4E	X57147, La Mantia et al. 1991	ERV9	TTTAGGGATAGCCCTCATCTGTTTGGTCAGGCCCTAGCCAA GATCTAGGCCACTTCTCAAGTCCAGGCACTCTGGTCCTTCA

4F	Herrmann 1998	SEQ49	TTCAGGGATATAGCCCCCATCTATTTGGTCAGGCATTAGCC CTTGAGCCAGTTCTCATACCTGGACACTCTGGTCCCTTTGG A
4G	Herrmann 1998	SEQ59	TTTAGGGATAGCCCTCATCTGTTTGGTCAGGCACAGGCCCA ATCTAGTTCACCTTCTCAAGTCCAGGCACTCTGGTTGTTCACT
4H	Herrmann 1998	SEQ60	TTTAGGGACAGCCCTCACTATTTCCGGTCAGGCACTTCAATT ACCTCTCCCAGCTACATCTNCNNCCYNGCATCTTGCTTCAG
4I	Herrmann 1998	SEQ63	TTCAGGGATAGCTCCCATCTATTTGGCCAGGCATTAACCCG ACTTAAGCCAGTTCTCATACGTGGACACTCTTGTCCTTTGGT
4J	Herrmann 1998	SEQ64	TTTAGAGATAGCCCTCACCTGTTTGGCCAAGCATTGGCCAA TTTAAGTCACTTCTTGACCCAGGTACCCTAATTCTTCAATA
4L	AF009668 Blond et al. 1999	HERV-W	TTCAGGGATAGCCCCCATCTATTTGGCCAGGCATTAGCCCA ACTTGAGTCAATTCTCATACCTGGACACTCTTGTCCTTCAGT
5A	AF020092 Seifarth et al. 1995	HERV-K (T47D)	CATGCTTAATAGTCCCACTATTTGTCAGTATTTTGTGGGGCG GCTTCAACCTGTCAGGGATCAGTTTCCCCGATGTTACATCG
5B	Herrmann 1998	SEQ05	ATGCTTAATAGTCCCACTATTTGTCAGTATTTTGTGGGGCGT CTTCAACCTGTCAGGGATCAGTTTCCCCGATGTTACATCGT
5C	Herrmann 1998	SEQ10	ATGCTTAATAGTCCCACTATTTGTCAGTATTTTGTGGGGGTG TTCAACCTGTCAGGGATTGAGTTTCCCCGATGTTACATCGTT
5E	U46939 Griffiths et al. 1997	SEQ U46939	ATGACTAACAGTCCTGCCATATGCCAGCTATATGTTGACCA GTAGAGCCTGTTCCGGCAGCAGTGCCCAAAAGTACAAATTTT
5F	U39937 Li et al. 1995	U39937	ATGCTTAATAGTCCAACTATTTGTCAGACTTTTGTAGGTCTGA CTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATT
5G	Herrmann 1998	SEQ35	AACCAGTATCAGGAGTTTTACAGCCAGGTAGTCAGGAGGAA AGTCATCCTGGTGCAGTGGAAGGGCATTGGATTTAAAGGC CT
5H	Herrmann 1998	SEQ36	AACAATGTTAGAATGGCTCACAGAACTCAGGAAAATACTTTA GTATTTAATGGTTTGTTACATAAGATACAACTCAAGGAACCA
5I	Herrmann 1998	SEQ41	TACCATGGACGACAAGCCTTCGTGTTACCACAAGGCACTGC G CAAGCATTGAATGTGATCGTTTGAGGGCAGGGTGATCGGG A
5J	Herrmann 1998	SEQ77	TGGAAGGGAGGACTTGAGCACATTCTTAAATGT GGCTCCTGTAATTTTAAACACATTGACACATGCTA
6A	U35161 Medstrand et al. 1993	HML-5	ATGCTGAACAGTCCTACCATGTGTCAGTAACATGTAAATCAA TTGCTCCCCAGTAGAAAATAATTTCTTAATTGCAAGATTATT

6E	HRU46939 Griffiths et al. 1997	HRV5	ATGACTAACAGTCCTGCCATATGCCAGCTATATGTTGACCA GTAGAGCCTGTTCCGGCAGCAGTGCCCCAAAAGTACAAATTTT
6F	Y07725	Foamy virus	TTTTTAAATAGTCCAGCATTGTTTACAGCTGATGTAGTAGAT CTAAAGAAATCCCTAATGTACAAGTGTATGTTGATGATATA
6G	Tuke et al. 1997	HTLV1	GTTTAAAAATAGTCCCACCCTGTTCGAAATGCAGCTGGCCC CCTGCAGCCCATTCCGGCAAGCTTTCCCCCAATGCACTATTC
6H	M10060, Shimotohno et al. 1985	HTLV2	GTTTAAAAACAGCCCCACCCTCTTCGAACAACAATTAGCAG CCTCAACCCCATGAGGAAAATGTTTCCACATCGACCATTG
6I	Tuke et al. 1997	HIV1	ATGGAAAGGATCACCAGCAATATTCCAAAGTAGCATGACAA CTTAGAGCCTTTTAAAAAACAAAATCCAGACATAGTTATCTA
6J	J04542	HIV2	TGGAAAGGATCACCAGCAATCTTTCATTCATGATGAGGCA TTAGAACCTTTCAGAAAAGCAAACCCAGACGTCAATTCTCATC
7A	U60269 Medstrand et al. 1997	HML-6	ATGCTTAACAGTCTTACGCTATGTCAGCATTTTGTAGGACAG TTAAAGAAGCCTCGGAATATGTTTCCTACTGCTTACATCATT
7B	Herrmann 1998	SEQ38	ATGCTCAACACCTACGTTAAGTCAGCATTTTGTAGGAAGAG T AAAGGACTCTCAGAATATGTTTCCCACTGCCTACATCGTTCA
7C	Herrmann 1998	SEQ56	ATGCTTAACAGCATTATATCAGCATGTTGTAGGATAGGCAT TAAA GGTGCCTCTGAATATGTTTCCCACAGCCTACATCCGTCATT T
7E	M15122 Moore et al. 1987	MMTV	ATGAAAAATAGCCCTACTTTATGTCAAAAATTTGTGGACAAA ATATTGACTGTAAGGGATAAATACCAAGACTCATATATTGTG
7F	AF038600 Akiyoshi et al. 1998	PERV	TTCAAGAACTCCCCGACCATCTTTGACGAAGCCCTACACAG CTGGCCAACTTCAGGATCCAACACCCTCAGGTGACCCTCCT G
7G	D10032 Kato et al. 1987	BaEV	TTCAAAAACCTCTCCCACTCTCTTCGATGAGGCTCTCCACAG CTCACCGACTTCCGGACCCAGCATCCAGAAGTGACCCTGC G
7H	M26927 Delassus et al. 1989	GaLV	TTCAAGAACTCTCCCACTCTCTTCGACGAGGCCCTCCACCG TTGGCTCCCTTTAGGGCCCTCAACCCCCAGGTGGTGTACT
7I	J02255 Van Beveren et al. 1981	MoMuLV	TTCAAAAACAGTCCCACCCTGTTTGATGAGGCACTGCACAG CTAGCAGACTTCCGGATCCAGCACCCAGACTTGATCCTGCT
7J	M12349 Sonigo et al. 1986	MPMV	ATGGCCAACAGTCCTACCTTATGTCAAAAATATGTGGCCAC ATACATAAGGTTAGACATGCCTGGAAACAAATGTATATTATA
8A	U07856, Dangel et al. 1994	HERV-KC4	ATGTTAAATAGTCCCACAGTTTGTCAAACTTTTGTAGGCAGA ATCCAGCCTGTTAGAGATCAGTTTCCAGATTTGTGCAGCAA



8B	Herrmann 1998	SEQ31	ATGTTAAACAGTTCCACAGTTTGTCAAACTTTTGTAGGCAAA ATCCAGCTAGTTAGAGATCAATTTCCAGATTGTTACATCATT
8E-8H	human genomic DNA	internal control	100 ng 10 ng 1 ng 0.1 ng
8I-8L	mixed oligo primers	internal control	100 pmol 10 pmol 1 pmol 0.1 pmol

Table 3.

## A. Human endogenous retroviral sequences

type-B retroviruses (HERV-K-superfamily)	HML-1 subgroup	HML1 (1A) Seq29 (1B*)
	HML-2 subgroup	HERV-K10 (2A) U87592 (2B)
	HML-3 subgroup	HML-3 (3A) S66676 (3B*) RT244 (3C*) Seq26 (3D*) Seq34 (4A*) Seq42 (4B*) Seq43 (4C*)
	HML-4 subgroup	HERV-K-T47D (5A) Seq05 (5B) Seq10 (5C)
	HML-5 subgroup	HML-5 (6A)
	HML-6 subgroup	HML-6 (7A) Seq38 (7B) Seq56 (7C)
	KC4 subgroup	HERV-K-C4 (8A) Seq31 (8B)
	not defined	U39937 (5F)
type-C retroviruses	HERV-H & related	AF026252 (2J) Seq61 (2K) Seq66 (2L)
	ERV9 & related	ERV9 (4E) Seq49 (4F) Seq59 (4G) Seq60 (4H) Seq63 (4I) Seq64 (4J)

	ERV-FRD	ERV-FRD (3E) Seq46 (3F)
	HERV-ERI family	HERV-E(4-1) (2H) Seq32 (2I)
	HERV-I & related	HERV-I (3H) HERV-IP-T47D (3I) Seq65 (3J)
	HERV-T	S71 pCRTK1 (2E) S71 pCRTK2 (2F)
	HERV-W	AF009668 (4L)
type-D retroviruses	MPMV related	Seq36 (5H)

Table 3 (continued)

Foamy virus related	HERV-L & related	G895836 (1E) Seq39 (1F) Seq40 (1G) Seq45 (1H) Seq48 (1I) Seq51 (1J) Seq58 (1K)
undefined retroviral elements		U46939 (5E) Seq35 (5G) Seq41 (5I) Seq77 (5J)
human non-viral retroposons		LINE-1 (3L)

B. Exogenous retroviruses

Human exogenous retroviruses		HRV5 (6E) Foamy virus (6F) HTLV-1 (6G) HTLV-2 (6H) HIV-1 (6I) HIV-2 (6J)
endogenous Mammalia retroviruses		MMTV (7E) PERV (7F) BaEV (7G) GaLV (7H) MoMuLV (7I) MPMV (7J)

## CLAIMS

1. A primer mixture ("MOP") consisting of forward and reverse primers for PCR, characterised in  
 that the forward and reverse primers are oligonucleotides,  
 that either ("MOP-ABD")  
 the forward primers exhibit the nucleotide sequences in accordance with SEQ ID NO. 1, namely:  
 ARAGTNYTDYCHCMRGGH, with a "head" at the 5' end and 3456 degenerations  
 and the reverse primers exhibit the nucleotide sequences in accordance with SEQ ID NO.2, namely:  
 NWDDMKDITYATCMAYRWA, with a "head" at the 5' end and 27648 degenerations,  
 or ("MOP-C")  
 the forward primers exhibits the nucleotide sequences in accordance with SEQ ID NO. 3, namely:  
 TKKAMMSKVYTRCYHCARGGG, with a "head" at the 5' end and 3072 degenerations and  
 the reverse primers exhibit the nucleotide sequences in accordance with SEQ ID NO. 4, namely:  
 MDVHDBRMDKYMAYVYAHKKA, with a "head" at the 5' end and 8192 degenerations  
 and the "head" stands for a nucleotide sequence which comprises an interface for a restriction enzyme and a clamp sequence at the 5' end of this interface and its length does not exceed half the length of the complete nucleotide sequence of the forward or reverse primer.
  
2. A primer mixture according to claim 1, characterised in that the "head" section of the nucleotide sequences presented in the sequence protocols has the nucleotide sequence GAAGGATCC.

3. A method for the specific detection and identification of retroviral nucleic acids/retrovirus in a specimen, characterised by type and sequence of the measures specified hereinafter:
  - Isolation of nucleic acids, namely DNA and/or RNA from the specimen,
  - Carrying out a PCR using the isolated DNA or an RT-PCR using the isolated RNA using one or both primer mixtures according to claim 1,
  - Purging the (RT)-PCR amplificates obtained and using these in an RDBH method using immobilised RDBH probes which each comprise (per probe) synthetic oligonucleotides whose nucleotide sequence corresponds to the retroviral nucleotide sequence of the retrovirus-specific reverse-transcriptase gene of the virus type to be detected with the relevant dot or a section of such a retroviral nucleotide sequence and exhibits no overlapping with the nucleotide sequences of the forward primer and the reverse primer of the primer mixtures used in the PCR or RT-PCR.
4. A method according to claim 3, characterised in that the nucleotide sequences of the synthetic oligonucleotides of the RDBH probes correspond to the retroviral nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L or a section of this region.
5. A method according to one of claims 3 or 4, characterised in that a mixture of equimolar quantities of both partners of a pair of synthetic oligonucleotides, which together correspond to a preferably 90 bp long section from the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L , is in each case used as immobilised RDBH probes.
6. A method according to claim 5, characterised in that both partners of the pair of synthetic oligonucleotides are approximately the same size or the same length, preferably approximately 45 bp long.

7. A use of one or several synthetic oligonucleotide(s) whose nucleotide sequence(s) correspond(s) with the nucleic acid region of a retrovirus-specific reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L or with a section of this nucleic acid region as reverse dot blot hybridisation probe(s) in a method according to one of claims 3 to 6.
8. A use of equimolar quantities of two synthetic oligonucleotides which together, positioned one after the other, correspond to a preferably 90 bp long section from the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L as reverse dot blot hybridisation probe(s) in a method according to one of claims 3 to 6.
9. A diagnosis kit for the specific detection and identification of retroviral nucleic acids/retroviruses in an arbitrary specimen, comprising at least one of the primer mixtures consisting of forward and reverse primers for the PCR according to claim 1 and at least one reverse dot blot hybridisation probe according to claim 7 or claim 8.

Figure 1

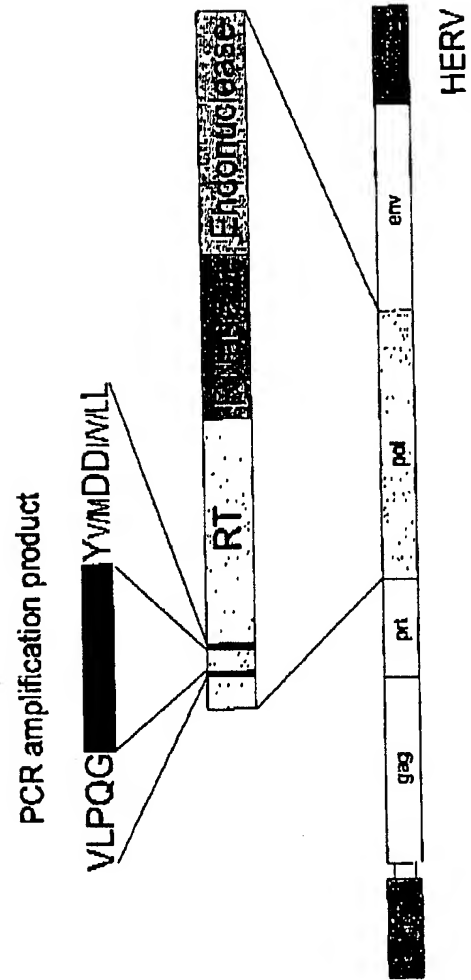
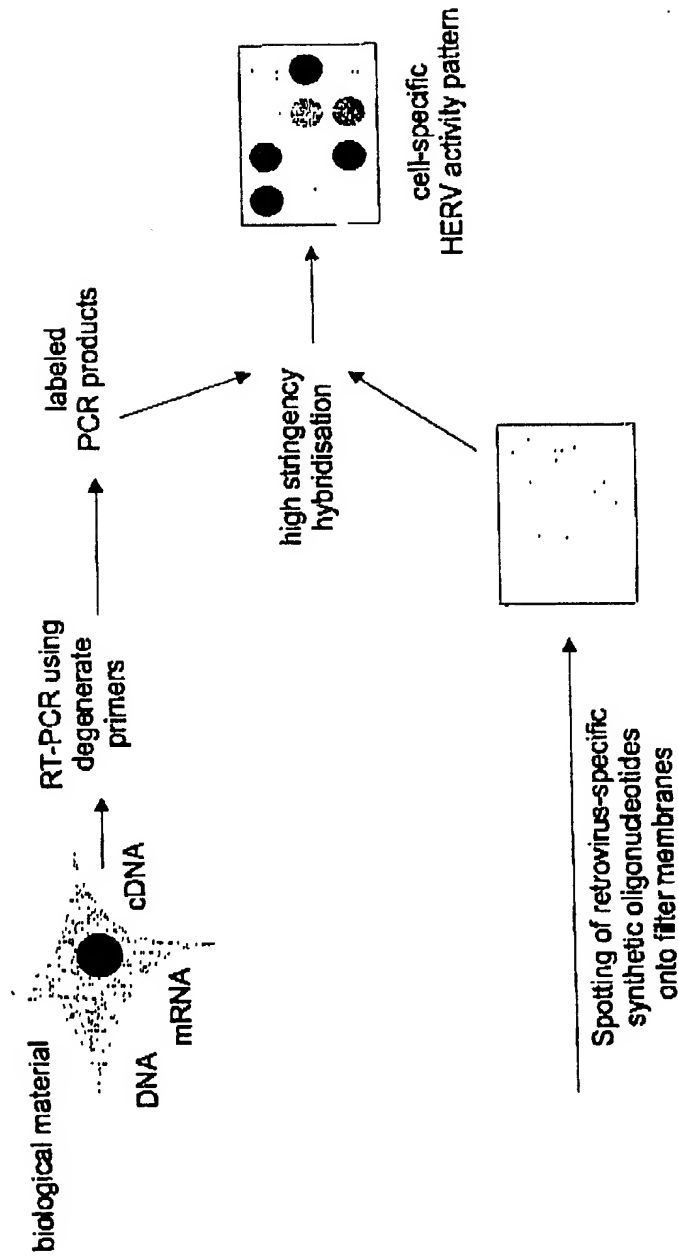


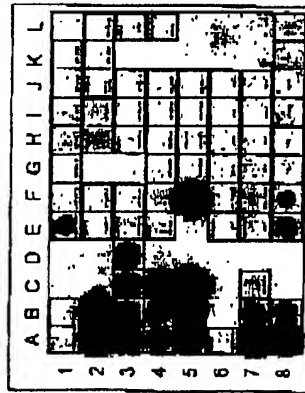
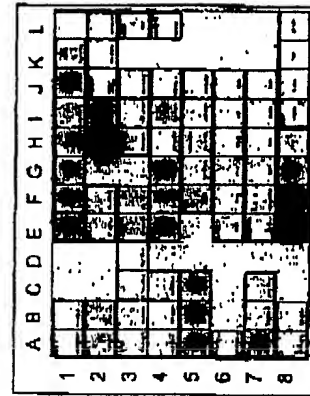


Figure 2



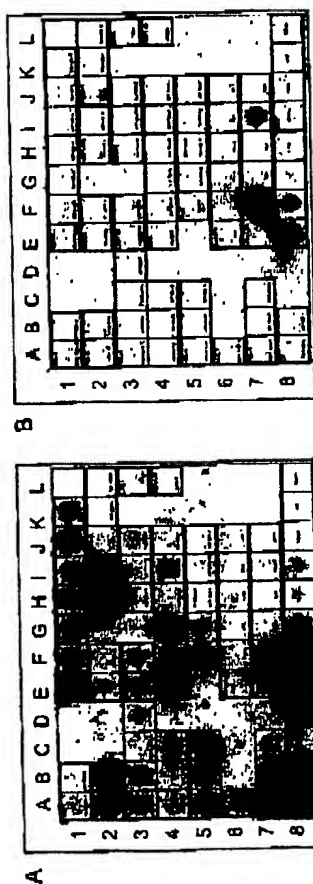
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**Figure 3**



10/009705

### Figure 4



# Declaration and Power of Attorney for Patent Application Erklärung für Patentanmeldungen mit Vollmacht German Language Declaration

Als nachstehend benannter Erfinder erkläre ich hiemit an Eides Statt:

daß mein Wohnsitz, meine Postanschrift, und meine Staatsangehörigkeit den im Nachstehenden nach meinem Namen aufgeführten Angaben entsprechen,

daß ich, nach bestem Wissen, der ursprüngliche, erste und alleinige Erfinder (falls nachstehend nur ein Name angegeben ist) oder ein ursprünglicher, erster und Miterfinder (falls nachstehend mehrere Namen aufgeführt sind) des Gegenstandes bin, für den dieser Antrag gestellt wird und für den ein Patent beantragt wird für die Erfindung mit dem Titel:

METHOD FOR THE SPECIFIC DETECTION AND IDENTIFICATION OF RETROVIRAL NUCLEIC ACIDS/RETROVIRUSES IN A SPECIMEN

deren Beschreibung  
(zutreffendes ankreuzen)

- ☐ hier beigelegt ist.  
☒ wurde angemeldet am 4 / April / 2000 unter der U.S.-Anmeldungs Nr. oder unter der Internationalen Anmeldenummer im Rahmen des Vertrags über die Zusammenarbeit auf dem Gebiet des Patentwesens (PCT) PCT/DE00/01071 und am \_\_\_\_\_ abgeändert (falls zutreffend).

Ich bestätige hiermit, daß ich den Inhalt der obigen Patentanmeldung einschließlich der Ansprüche durchgesehen und verstanden habe, die eventuell durch einen Zusatzantrag, wie oben erwähnt, abgeändert wurde.

Ich erkenne meine Pflicht zur Offenbarung irgendwelcher Informationen an, die für die Prüfung der vorliegenden Anmeldung in Einklang mit Titel 37, Code of Federal Regulations, §1.56 von Belang sind.

Ich beanspruche hiemit ausländische Prioritätsvorteile gemäss Titel 35, US-Code, §119(a)-(d), bzw. §365(b) aller unten angegebenen Auslandsanmeldungen für ein Patent oder Erfinderurkunden, oder §365(a) aller PCT internationalen Anmeldungen, welche wenigstens ein Land ausser den Vereinigten Staaten von Amerika benennen, und habe nachstehend durch ankreuzen, sämtliche Auslandsanmeldungen für Patente, oder Erfinderurkunden oder PCT internationale Anmeldungen angegeben, deren Anmeldetag dem der Anmeldung, für welche Priorität beansprucht wird, vorangeht.

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR THE SPECIFIC DETECTION AND IDENTIFICATION OF RETROVIRAL NUCLEIC ACIDS/RETROVIRUSES IN A SPECIMEN

the specification of which  
(check one)

- ☐ is attached hereto  
☒ was filed on 4 / April / 2000 as United States Application Number or PCT International Application Number PCT/DE00/01071 and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications  
(Frühere ausländische Anmeldungen)

Priority Claimed?  
Priorität beansprucht?

199 21 419.0 (Number) (Nummer)	Germany (Country) (Land)	8/May/1999 (Day/Month/Year Filed) (Tag/Monat/Jahr eingereicht)	<input checked="" type="checkbox"/> Yes Ja	<input type="checkbox"/> No Nein
_____ (Number) (Nummer)	_____ (Country) (Land)	_____ (Day/Month/Year Filed) (Tag/Monat/Jahr eingereicht)	<input type="checkbox"/> Yes Ja	<input type="checkbox"/> No Nein

Ich beanspruche hiermit gemäss Titel 35, US-Code, §119(e), den Vorzug aller unten aufgeführten US-Hilfsanmeldungen

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) below

(Application No. / Anmeldnr.)

(Filing Date / Anmeldedatum)

(Application No. / Anmeldnr.)

(Filing Date / Anmeldedatum)

Ich beanspruche hiermit gemäss Titel 35, US-Code, §120, den Vorzug aller unten aufgeführten US-Patentanmeldungen bzw. §365(c) aller PCT internationalen Anmeldungen, welche die Vereinigten Staaten von Amerika benennen, und erkenne, insofern der Gegenstand eines jeden früheren Anspruchs dieser Patentanmeldung, bzw. PCT Internationalen Anmeldung in einer gemäß dem ersten Absatz von Titel 35, US-Code §112 vorgeschriebenen Art und Weise offenbart wurde, meine Pflicht zur Offenbarung jeglicher Informationen an, die zur Prüfung der Patentfähigkeit in Einklang mit Titel 37, Code of Federal Regulations, §1.56 von Belang sind und im Zeitraum zwischen dem Anmeldedatum der früheren Patentanmeldung und dem nationalen oder im Rahmen des Vertrags über die Zusammenarbeit auf dem Gebiet des Patentwesens (PCT) gültigen internationalen Anmeldedatum bekannt geworden sind.

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Appl. No.)  
(Anmeldnr.)

(Filing Date)  
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(Status)  
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Ich erkläre hiermit, daß alle von mir in der vorliegenden Erklärung gemachten Angaben nach meinem besten Wissen und Gewissen der vollen Wahrheit entsprechen, und daß ich diese eidesstattliche Erklärung in Kenntnis dessen abgebe, daß wissentlich und vorsätzlich falsche Angaben gemäss §. 1001, Titel 18 US-Code strafbar sind und mit Geldstrafe und/oder Gefängnis bestraft werden können, und daß derartige wissentlich und vorsätzlich falsche Angaben die Rechtswirksamkeit der vorliegenden Patentanmeldung oder eines darauf erteilten Patentes gefährden können.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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Unterschrift des Erfinders

Datum

Inventor's Signature

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## SEQUENZPROTOKOLL

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